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Award Number: DAMD17-00-1-0544

TITLE: Signal Transduction Targets for Pharmacological Intervention in Type I Neurofibromatosis

PRINCIPAL INVESTIGATOR: Raymond R. Mattingly, Ph.D., Richard A. Gibbs, Ph.D., John J. Reiners Jr., Ph.D., Michael A. Tainsky, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, Michigan 48202

REPORT DATE: November 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20020416 117

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 074-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	November 2001	Annual (1 Oct 00 - 1 Oct 01)	
4. TITLE AND SUBTITLE		5. FUNDING NUMBERS	
Signal Transduction Targets for Pharmacological Intervention in Type I Neurofibromatosis		DAMD17-00-1-0544	
6. AUTHOR(S)			
Raymond R. Mattingly, Ph.D., Richard A. Gibbs, Ph.D., John J. Reiners Jr., Ph.D., Michael A. Tainsky, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)		8. PERFORMING ORGANIZATION REPORT NUMBER	
Wayne State University Detroit, Michigan 48202 E-Mail: r.mattingly@wayne.edu			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) This New Investigator Award has the objective of the identification of new pharmacological approaches to NF1 treatment. We will proceed through the following aims and hypotheses: 1. Role of the Ras/MAP kinase pathway in neurofibromin-deficient cells. Our hypothesis is that activation of the Ras/MAP kinase signaling system plays a critical role in the phenotypes of human neurofibrosarcomas. The experiments use 2 novel pharmacological approaches to inhibit the Ras/MAP kinase pathway: 3-allylfarnesol [a novel inhibitor of protein farnesylation] and PD184352 [a new-generation inhibitor of MEK1]. We assay a variety of end-points in the cells, including the level of Ras-GTP loading, activation status of ERK MAP kinases, transcriptional reporter assays of Ras/MAP kinase-responsive genes, and rates of cellular proliferation. 2. Role of Rac/Rho family small GTPases in neurofibromin-deficient cells. We will examine the hypothesis that Rac/Rho small GTPases contribute to the NF1 phenotype by use of novel, selective inhibitors of protein geranylgeranylation, such as 3-allylgeranylgeraniol. These experiments should validate the use of two new classes of pharmacological inhibitors of the Ras/MAP kinase pathway in fibroblast systems that have aberrant Ras activation due to neurofibromin-deficiency. This project will thus lead to the identification of relatively non-toxic and mechanistically specific drugs, which could then be investigated in future clinical trials for chemoprevention of tumors and chemotherapy of malignancies in NF1 patients.			
14. SUBJECT TERMS NF1, neurofibrosarcoma, ras, prenylation, MEK			15. NUMBER OF PAGES 16
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

This New Investigator Award has the objective of the identification of new pharmacological approaches to NF1 treatment. We will proceed through the following aims and hypotheses:

1. **Role of the Ras/MAP kinase pathway in neurofibromin-deficient cells.** Our hypothesis is that activation of the Ras/MAP kinase signaling system plays a critical role in the phenotypes of human neurofibrosarcomas. The experiments use 2 novel pharmacological approaches to inhibit the Ras/MAP kinase pathway: 3-allylfarnesol [a novel inhibitor of protein farnesylation] and PD184352 [a new-generation inhibitor of MEK1]. We assay a variety of end-points in the cells, including the level of Ras-GTP loading, activation status of ERK MAP kinases, transcriptional reporter assays of Ras/MAP kinase-responsive genes, and rates of cellular proliferation.
2. **Role of Rac/Rho family small GTPases in neurofibromin-deficient cells.** We will examine the hypothesis that Rac/Rho small GTPases contribute to the NF1 phenotype by use of novel, selective inhibitors of protein geranylgeranylation, such as 3-allylgeranylgeraniol.

These experiments should validate the use of two new classes of pharmacological inhibitors of the Ras/MAP kinase pathway in fibroblast systems that have aberrant Ras activation due to neurofibromin-deficiency. This project will thus lead to the identification of relatively non-toxic and mechanistically specific drugs, which could then be investigated in future clinical trials for chemoprevention of tumors and chemotherapy of malignancies in NF1 patients.

Body of Report: Arranged by Approved Statement of Work

Task 1 To establish the effects of novel pharmacological inhibitors of the Ras/MAPk pathway in NF1 model cell culture systems from the NF1 transgenic mouse and human neurofibrosarcoma lines (months 1-24)

- a. *Prepare mouse embryo fibroblast (harvested on day 12.5 as lethality of homozygous embryos occurs by day 14.5) populations of NF1^{+/+}, NF1^{+/-}, and NF1^{-/-} genotypes (months 1-12)*

This work has not yet been performed. Subsequent to the grant application submission (September 1999), 2 seminal papers were published in December 1999:

- Mouse models of tumor development in Neurofibromatosis Type 1, K. Cichowski et al., Science 286:2172 (1999)
- Mouse tumor model for Neurofibromatosis type 1. Vogel et al., Science 286:2176 (1999).

Both these manuscripts emphasize that the NF1-deficient mouse that we had planned to use is much less relevant to a model for human disease than an NF1^{+/-} cross with a p53-deficient line. Since our institutional animal protocol does not cover this variation in experimental design, we are in the process of amending our submission to the Institutional Review Board to allow these experiments to proceed.

In the interim while progress on this task is delayed, we have made enhanced efforts on the other tasks of our Grant.

- b. Characterize the phenotypes of the NF1 fibroblasts and the human neurofibrosarcoma lines with respect to Ras-GTP levels, ERK activity, transcriptional events, and cellular proliferation (months 7-18)

We have made significant progress in establishing the phenotypes of 2 NF1-deficient human neurofibrosarcomas (lines ST88-14 and NF90-8) together with a control NF2-related tumor (ST5-26T). All of these lines were generously provided by Prof. T. Glover of the University of Michigan.

It is clear that the 2 NF1-deficient lines have elevated levels of Ras.GTP when compared to the control NF2 line (Figure 1). The high levels of Ras.GTP are well maintained even following overnight deprivation from serum stimulation (compare lanes labeled "U" and "C" in the figure). Indeed, this constitutive level of Ras activity is greater than the degree to which Ras.GTP levels can be stimulated by acute treatment with serum in the control line (26T cells lane labeled "S"). Note that the total content of Ras protein is not affected by neurofibromin deficiency. We have also found correspondingly high levels of MAP kinase activity in the ST88-14 and NF90-8 lines (see below). These results, therefore, strongly support our hypothesis that an over-active Ras signal transduction pathway is present in cells that are deficient in neurofibromin, and thus that pharmacological inhibitors of this signalling may target the NF1 phenotype. Further experiments will confirm these results and extend these studies into reporters of Ras-dependent transcriptional activity.

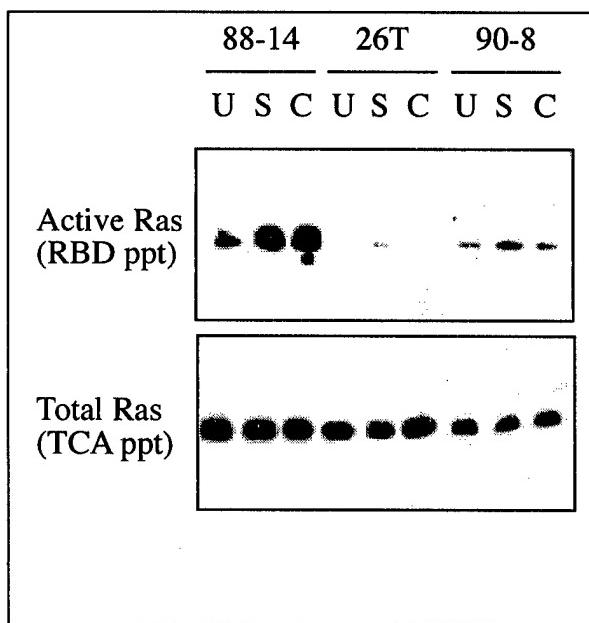


Figure One: Constitutive Activation of Ras in Neurofibromin-deficient Cells. Human tumor cell lines that are deficient in neurofibromin (88-14 and 90-8) or control (26T) were cultured in full growth media with 10% fetal calf serum ("C"), or starved of serum overnight prior to experiment ("U", "S"). Serum was then added back for 5 minutes as shown ("S"). The cells were lysed and protein measured by modified Lowry assay so that equal amounts were subject to further analysis. One third was taken for TCA precipitation to establish the total amount of Ras present (lower panels). Activated Ras (Ras.GTP) was isolated from the remainder of the lysate by affinity pulldown with the Ras binding domain of Raf protein kinase that had been immobilized to glutathione-Sepharose beads through its fusion to GST (upper panel). The results shown are western blots with a monoclonal antibody directed against Ras. The results shown are representative of 3-5 independent experiments.

We have also established several experimental approaches to study the proliferation of these neurofibrosarcoma lines:

- Anchorage-independent growth can be assayed through division in soft agar
- DNA synthesis can be measured through uptake of bromodeoxyuridine
- Progress through the cell cycle can be measured by propidium iodide staining for DNA content and sorting by FACS analysis.

All of these techniques will now be available for study of the lines in the next tasks.

c. Characterize the effects of 3-allylFOH and PD184325 on the phenotypes of the NF1 fibroblast and neurofibrosarcoma lines (months 13-24)

Although these experiments were not due to start until the current year, we have made early progress on these tasks by combining them with the characterization studies for task "1b". The MEK inhibitor, PD184352, has been particularly interesting. It is very active in the inhibition of both the (elevated) basal MAP kinase activity present in ST88-14 and NF90-8 cells, and in preventing the activation of MAP kinase by additional stimulation from phorbol diester treatment (Figure 2; note the substantial signal of active MAPk in the 1st lanes that represent serum-starved, neurofibromin-deficient cells without additional stimulation). PD184352 is clearly more potent as an inhibitor when used in medium with reduced serum content.

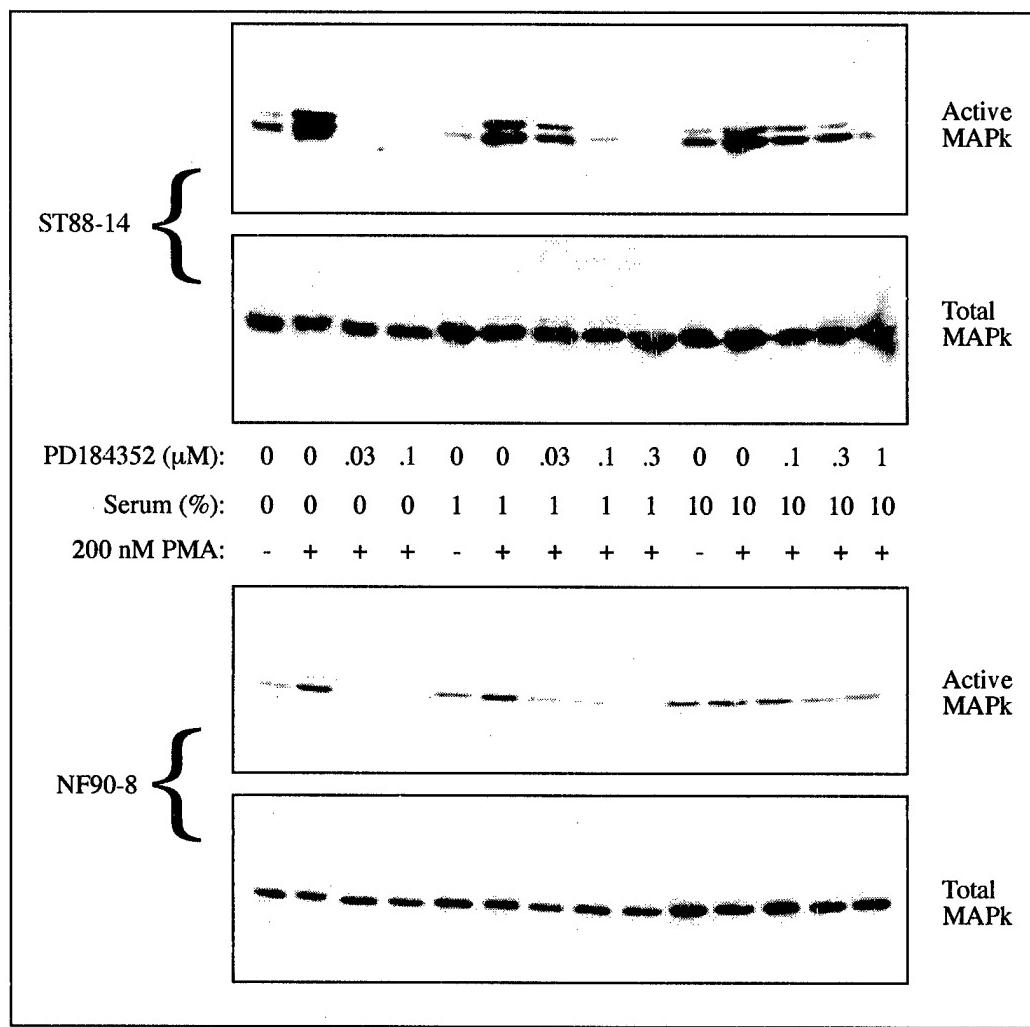


Figure Two:
Inhibition of
MAP kinase
activation by
the MEK
inhibitor,
PD184352.
Confluent plates of
neurofibrosarcoma
cells were shifted
to media with the
indicated
concentration of
fetal calf serum for
24 hours prior to
experiment. The
plates were treated
with PD184352 for
1 hour as shown,
and then
stimulated with or
without 200 nM
phorbol 12-
myristate 13-
acetate (PMA) for 5
minutes. Whole
cell lysates were
then prepared in
boiling Laemmli
sample buffer and
subjected to
western blotting
with an antibody
that recognizes

the active, dually-phosphorylated form of MAP kinase (MAPk) (*upper panels*). The blots were then stripped and reprobed with an antibody that recognizes MAPk whether phosphorylated or not (*lower panels*). Further details of these methods are in the paper in the Appendix (Mattingly *et al.*, 2001).

This assay protocol was developed from that used in a recent neuroblastoma project, with the final neuroblastoma samples providing positive controls during the start-up period of this Neurofibromatosis study. The contribution of the projects to each other is acknowledged in the support section of the paper that reports the results from the neuroblastoma work (see below).

In agreement with its ability to suppress MAP kinase activity, PD184352 was able to inhibit the anchorage-independent growth of ST88-14 cells with an $IC_{50} = 0.82 \mu\text{M}$. Interestingly, this value is similar to that required for inhibition of the growth in soft agar of the control ST5-26T line ($IC_{50} = 0.33 \mu\text{M}$).

Task 2 To establish the effects of 3-allylGGOH on the NF1 fibroblasts and human neurofibrosarcoma lines (months 1-24)

a. Establish that 3-allylGGOH is active in inhibition of prenylation in these systems (months 1-12)

Unfortunately, we have yet to obtain convincing evidence that 3-allylGGOH is an effective inhibitor of prenylation in our neurofibromin-deficient cells. Figure 3 shows data for the marker protein Rap1, which we are using to monitor protein prenylation, in ST88-14 cells that have been treated with 4 inhibitors of protein prenylation: GGT1-286 (a commercially-available inhibitor of GGTase from Calbiochem), 2-cisGGOH and 3-allylGGOH (2 novel inhibitors of GGTase that have been shown to be effective in other systems) and 3-allylFOH (an inhibitor of FTase that was used here as a negative control). We have added GGTI-286 to our experimental design to provide a positive control in these studies and so to aid in the definition of conditions under which geranylgeranylation may be inhibited.

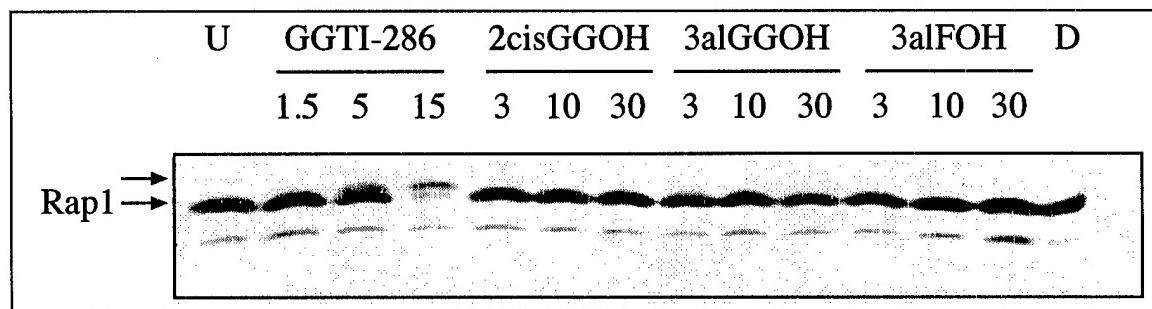


Figure Three: Inhibition of Protein Prenylation in ST88-14 Neurofibrosarcoma Cells. Sub-confluent ST88-14 cells were cultured for 48 hours in fresh growth medium plus the agents shown (concentrations are μM , "U" is untreated control, "D" is DMSO vehicle control). Whole cell lysates were prepared in boiling Laemmli sample buffer and subjected to western blotting with a polyclonal antibody directed against Rap1 (Santa Cruz). The unprocessed form of Rap1 is revealed as a more slowly migrating band (upper arrow) that is present when geranylgeranylation is inhibited.

It is clear that while the GGTI-286 causes an upshift in the Rap1 protein band that is indicative of a block of its processing, the 2-cisGGOH and 3-alGGOH do not. We are currently testing whether the combination of lovastatin (to reduce the size of the intracellular pools of prenylation precursors) may potentiate the effects of 3-allylGGOH and 2-cisGGOH on protein prenylation. In parallel experiments, we are also defining conditions under which the farnesylation of Ras may be blocked by 3-alFOH (with and without co-treatment with lovastatin). We are also developing an alternative assay protocol that separates the soluble, cytosolic (unprocessed) Rap1 (and Ras) from the membrane-bound, prenylated Rap1 (and Ras). We expect that this assay may increase our resolution of the effects of the inhibitors.

b. Characterize the effects of 3-allylGGOH on the phenotypes of the systems (months 7-24)

In agreement with the lack of obvious effect of 3-allylGGOH and 2-cisGGOH on protein prenylation, we find that they exhibit little inhibition of cell division in the anchorage-independent growth assay ($IC_{50} > 20 \mu M$), and little effect of distribution of cells in the phases of the cell cycle (data not shown). We will revisit these assays with combinations of inhibitors with lovastatin if such treatments are effective in blocking the processing of Rap1. We are also now using the GGTI-286 inhibitor in assays of growth in soft agar and cell cycle analyses to provide positive controls for the effect of an inhibitor of geranylgeranylation on these parameters.

Key Research Accomplishments

- Demonstration of activated Ras and MAP kinase pathway in human neurofibrosarcomas that are deficient in neurofibromin
- Establishment of conditions for assay of the effects of pharmacological inhibitors of signal transduction on the growth of neurofibrosarcomas.
- Correlation of the effect of the MEK inhibitor, PD184352, to reduce active MAP kinase and inhibit anchorage-independent cell growth of the ST88-14 human neurofibrosarcoma.
- Identification of GGTI-286, an inhibitor of GGTase, as an effective tool for the block of Rap1 processing in neurofibrosarcomas.

Reportable Outcomes

"Down-regulation of growth factor-stimulated MAP kinase signaling in cytotoxic drug-resistant human neuroblastoma cells" by Raymond R. Mattingly, Michelle L. Milstein, and Bernard L. Mirkin, *Cellular Signalling*, 13:499-505 (2001).

Conclusions

We have made significant progress in testing our hypothesis that aberrant activation of the Ras/MAP kinase pathway plays a critical role in the transformed phenotype of neurofibromin-deficient cells. There is now clear evidence that neurofibromin-deficient human neurofibrosarcomas have elevated levels of active Ras and active MAP kinase. Further, we have shown that reduction of the level of active MAP kinase by inhibition of the upstream kinase MEK is associated with an inhibition of the ability of the neurofibrosarcoma cells to grow in soft agar. Extension of these results into the NF1^{-/-} mouse system has been delayed since it is now clear that better models are available by crossing into a p53-deficient model (Cichowski et al., 1999; Vogel et al., 1999).

Our second hypothesis, that Rac and Rho proteins contribute to the phenotype of neurofibromin-deficient cells, is in the process of being tested. Our initial attempts to use analogues of the geranylgeraniol substrate as inhibitors of the GGTase-dependent processing of small GTPases have not been successful. We will combine these agents with lovastatin to try to increase their efficacy, and also test an analogue of the peptide substrate of GGTase, termed GGTI-286, which appears to be an effective inhibitor of Rap prenylation in human neurofibrosarcomas.

We continue to pursue our goal of the identification of relatively non-toxic inhibitors of the Ras/MAP kinase signal transduction in neurofibromin-deficient cells so that additional agents

will be available for future clinical trials for chemoprevention of tumors and therapy of malignancies in NF1 patients.

References

K. Cichowski et al., Mouse models of tumor development in Neurofibromatosis Type 1. Science 286:2172 (1999)

Vogel et al., Mouse tumor model for Neurofibromatosis Type 1. Science 286:2176 (1999).

Appendix

"Down-regulation of growth factor-stimulated MAP kinase signaling in cytotoxic drug-resistant human neuroblastoma cells" by Raymond R. Mattingly, Michelle L. Milstein, and Bernard L. Mirkin, *Cellular Signalling*, 13:499-505 (2001).

Down-regulation of growth factor-stimulated MAP kinase signaling in cytotoxic drug-resistant human neuroblastoma cells

Raymond R. Mattingly^{a,b,*}, Michelle L. Milstein^a, Bernard L. Mirkin^c

^aDepartment of Pharmacology, Wayne State University, Detroit, MI 48201, USA

^bProgram in Molecular Biology and Genetics, Barbara Ann Karmanos Cancer Institute, Detroit, MI 48201, USA

^cDepartments of Pediatrics and Molecular Pharmacology and Biological Chemistry, Children's Memorial Institute for Education and Research, Chicago, IL 60614, USA

Received 21 January 2001; accepted 21 February 2001

Abstract

The mitogen-activated protein kinase (MAPk) signaling pathway, which plays a critical role in the proliferation of mammalian cells, is frequently up-regulated in human tumors and may contribute to the transformed phenotype. Since a major limitation of current cancer chemotherapy is prevalent resistance to cytotoxic drugs, this study determined whether alterations in growth factor signaling through MAPk may contribute to this phenomenon in human neuroblastoma cell lines. Drug-resistant SKNSH cell lines were established by long-term incubation with increasing concentrations to 10^{-6} M doxorubicin (SKNSH rDOX6) or MDL 28842 (SKNSH rMDL6). The expression of epidermal growth factor receptor (EGFR) and epidermal growth factor (EGF)-induced EGFR tyrosine phosphorylation were lower in drug-resistant SKNSH cells than their wild-type counterparts. In SKNSH rDOX6 cells, decreased activation and reduced nuclear translocation of MAPk in response to EGF, or lysophosphatidic acid (LPA), or phorbol 12-myristate 13-acetate (PMA), were observed. In SKNSH rMDL6 cells, although MAPk could be activated to wild-type levels by ligand stimulation, the translocation of active MAPk to the nucleus was also reduced. These results suggest that resistance to cytotoxic drugs in human neuroblastoma cell lines is associated with a decrease in growth factor signaling through the MAPk pathway. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Epidermal growth factor; Lysophosphatidic acid; ERK; Nuclear localization; Doxorubicin; Confocal immunofluorescence

1. Introduction

A major pathway for regulation of cellular growth and differentiation is through the ubiquitous mitogen-activated protein kinase (MAPk) cascade that transmits growth factor

signals from the cell membrane to the nucleus [1]. This evolutionarily conserved signaling pathway is thought to be the principal mechanism through which signals from both polypeptide growth factor receptors with intrinsic tyrosine kinase activity and receptors coupled to heterotrimeric G proteins stimulate cell cycle progression and proliferation [2]. Oncogenic Ras proteins [3], overexpressed growth factor receptors [4], and enhanced autocrine growth factor loops [5] are commonly found in human tumors, and can induce constitutive and inappropriate activation of MAPk [6]. Thus pharmacological intervention in the MAPk cascade has been identified as a promising new approach to cancer therapy [7].

A major limitation in response to current chemotherapy of human cancers is the widespread occurrence of inherent or acquired resistance to cytotoxic drugs [8]. In certain cases, including neuroblastoma [9,10], altered growth factor signaling has been suggested to provide chemoprotection [11,12]. Modulation of growth factor receptors

Abbreviations: MAPk, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK1, MAPk/ERK kinase 1; EGFR, epidermal growth factor receptor; SKNSH, human neuroblastoma cell line; rDOX6, cells resistant to 1 μM doxorubicin; rMDL6, cells resistant to 1 μM MDL 28842; LPA, lysophosphatidic acid; PMA, phorbol 12-myristate 13-acetate; NGF, nerve growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium

* Corresponding author. Department of Pharmacology, Wayne State University, 540 East Canfield Avenue, Detroit, MI 48201, USA. Tel.: +1-313-577-6022; fax: +1-313-577-6739.

E-mail address: r.mattingly@wayne.edu (R.R. Mattingly).

in neuroblastoma tumor cells has been shown to occur under a variety of clinical [13–15] and experimental [16,17] conditions. In murine neuroblastoma models, homologous and heterologous down-regulation of growth factor receptors occurs following overexpression of nerve growth factor (NGF). Specifically, retroviral infection with the NGF gene induces down-regulation of the NGF receptor in C-1300 neuroblastoma cells and of the epidermal growth factor (EGF) receptor in Neuro-2A neuroblastoma cells [12]. In both cases there is a marked decrease in cell replication and proliferation rate following transfer of the NGF gene. Neuroblastoma cells that become drug-resistant also manifest a decrease in proliferation rate, suggesting that changes in the expression or activation of components of growth factor signaling pathways may be associated with failure to respond to cytotoxic agents [12,18,19].

The current study has determined the relationship that exists between acquired drug resistance in human neuroblastoma cells to doxorubicin, a topoisomerase-2 inhibitor [20], and MDL-28842, a mechanism-based inhibitor of S-adenosylhomocysteine hydrolase [21–23], the expression of epidermal growth factor receptor (EGFR), and the activation and nuclear translocation of MAPk.

2. Materials and methods

2.1. Cell culture

SKNSH cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The generation of resistant lines was performed by incubating cells with stepwise increments of drugs from concentrations of 10^{-9} to 10^{-6} M. Cytotoxicity was assessed by determining cell survival with 3-(4,5-dimethyl-2-thiazoyl)2,5-diphenyl tetrazolium bromide.

2.2. Western blots

Western blots for the EGFR and phosphotyrosine were performed by standard procedures using cell lysates that had been prepared in a lysis buffer consisting of 1% Triton X-100, Tris buffer, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, 0.15 U aprotinin/ml, and 1 mM sodium orthovanadate. The samples were applied to a 7.5% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) gel for electrophoresis. Proteins were transferred from the gel to Immobilon-P membranes. EGFR and phosphotyrosine containing proteins were detected with a primary anti-EGFR or anti-phosphotyrosine monoclonal antibody and a horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence. The electrophoretic gels were analyzed using SigmaGel gel analysis software (SPSS) after image scanning.

For MAPk assays, cells were passaged onto 35-mm culture dishes and grown for 48 h to confluence. The monolayers were washed twice with serum-free DMEM and then incubated in this medium for a further 48 h. After agonist treatments as shown in the figures, the media was removed and 100 μ l of boiling sample buffer (75 mM Tris-HCl pH 6.8, 1.5% w/v SDS, 7.5% w/v glycerol, 200 mM β -mercaptoethanol, 0.03% w/v bromophenol blue, 0.003% w/v pyronin-Y) was added. The lysates were scraped into microfuge tubes and boiled for a further 5 min prior to separation on 10% SDS/PAGE gels and transfer to nitrocellulose. Detection of active extracellular signal-regulated kinase (ERK) MAPk was performed with a 1:2000 dilution of anti-diphosphorylated ERK-1 and -2 (Sigma) followed by a 1:20,000 dilution of anti-mouse conjugated to horse radish peroxidase (Santa Cruz) and enhanced chemiluminescence. The membranes were then stripped by incubation for 30 min in 62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 100 mM β -mercaptoethanol at 70°C, and then extensively washed. Total MAPk was then assayed by incubation in a 1:5000 dilution of anti-ERK (Transduction), followed by secondary antibody and detection as before. X-ray films were scanned using a Molecular Dynamics Storm imaging system and quantified using ImageQuant software.

2.3. Confocal immunofluorescence

Cells were passaged onto 60-mm culture dishes that contained a glass cover slip and grown to subconfluence. The media was removed and the cells were washed twice with serum-free DMEM and then incubated in this medium for an additional 48 h. Specific agonists were added (as shown in the figures) and the media was removed. The cells were washed twice with cold phosphate-buffered saline (PBS), and fixed in a fresh 4% w/v solution of paraformaldehyde (Fisher) in PBS for 10 min at room temperature. The fixed cells were washed with PBS, incubated in 50 mM NH₄Cl in PBS, washed again in PBS, and then permeabilized by incubation for 5 min in 0.5% w/v Triton X-100 in PBS. After further washing, the cells were blocked in 3% bovine serum albumin (BSA) in PBS. Active ERK MAPk were detected by a 1:250 dilution of anti-diphosphorylated ERK-1 and -2 (Sigma) followed by a 1:250 dilution of anti-mouse conjugated to Cy3 (Jackson). Total MAPk was detected using a 1:50 dilution of anti-ERK (Transduction). In some experiments, 0.5 mg/ml 4,6-diamidino-2-phenylindole (DAPI) was included in the first wash after the secondary antibody to stain the nuclei.

2.4. Reagents

Stock solutions of EGF (Gibco) were prepared in 100 μ g/ml BSA. Stock solutions of phorbol 12-myristate 13-acetate (PMA, Sigma) were prepared in dimethylsulfoxide. Stock

solutions of lysophosphatidic acid (LPA, Sigma) were prepared in water.

3. Results

3.1. EGFR expression and tyrosine kinase activity in drug-resistant SKNSH neuroblastoma cells

Drug-resistant SKNSH neuroblastoma cell lines were generated by sustained, incremental incubation up to 1 μ M doxorubicin (SKNSH rDOX6) or MDL 28842 (SKNSH rMDL6). The lines exhibited alterations in cellular morphology, substrate attachment, and reduced cellular proliferation rates that persisted even after subculturing in medium that lacked the drugs. The doubling time of wild-type cells was 49 h, whereas that of SKNSH rMDL6 and SKNSH rDOX6 lines were 63 and 92 h, respectively. Corollary investigations demonstrate that the drug-resistant cells were also less responsive to the stimulatory effects of EGF on cell proliferation [24].

In order to determine whether alterations in the expression of EGFR were associated with these phenotypic changes, Western blots were performed on wild-type and drug-resistant cells (Fig. 1A). A marked reduction in the level of EGFR expression was observed in both resistant lines, with a greater decrease in the SKNSH rDOX6 cells. To assess the functionality of the EGFR, its ability to undergo (auto)phosphorylation on tyrosine residues following exposure to EGF was assessed (Fig. 1B). Ligand-induced phosphorylation was abolished in the drug-resistant cells when compared to wild-type cells.

3.2. Effects of EGF, phorbol ester, and LPA on MAPk activation in SKNSH rDOX6 and SKNSH rMDL6 cells

The ability of EGF to stimulate the activation of MAPk in wild-type and drug-resistant neuroblastoma cells was assessed by Western blotting. An antibody [25] that selectively recognized the active dually phosphorylated forms of p44 and p42 MAPk (which are also called ERK-1 and -2, respectively) was utilized. EGF (10 ng/ml) activated MAPk in wild-type and SKNSH rMDL6 cells, but not in SKNSH rDOX6 cells (Fig. 2A and B). This inability to activate was not due to a relative shift in the potency of EGF, as even concentrations up to 175 ng/ml do not have effect in the SKNSH rDOX6 cells (data not shown). Immunoblots performed with an antibody that recognized both phosphorylated and dephosphorylated MAPk demonstrated that the expression of p42 MAPk in resistant cells was equivalent to wild-type levels. Since SKNSH rMDL6 cells also manifest a reduction in EGFR expression and phosphorylation relative to wild-type cells (Fig. 1), the effect of EGF on MAPk activation was examined in these cells in more detail. Concentration-response and kinetic studies (Fig. 3) revealed that EGF was extremely potent in activating

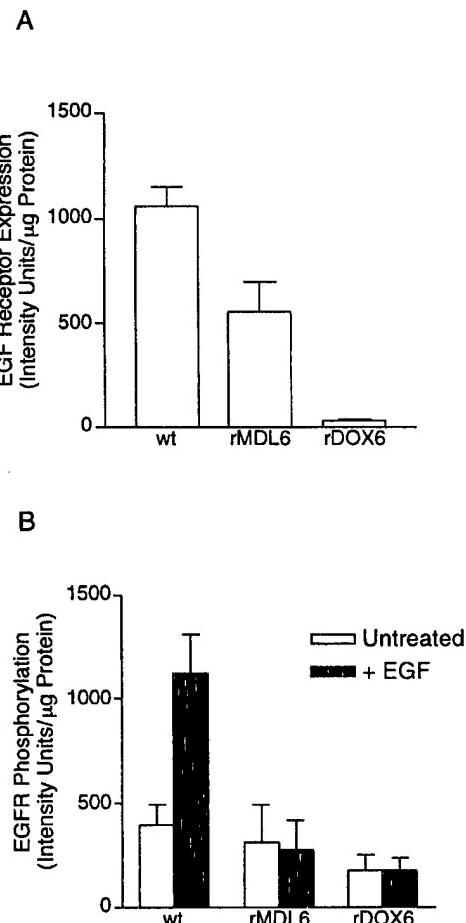


Fig. 1. Expression and activation of the EGFR in wild-type and drug-resistant SKNSH cells. (A) Expression of the EGFR in the human neuroblastoma cell line designated SKNSH (wt) and variants that were resistant to either MDL 28842 (rMDL6) or to doxorubicin (rDOX6). The epidermal carcinoma cell line A431 [4], which constitutively over-expresses EGFR, was used as a standard to identify the 170-kDa band that represents the receptor (not shown). (B) Tyrosine phosphorylation of the EGFR in wild-type and drug-resistant SKNSH cells. Cells were stimulated for 1 min with 10 ng/ml EGF. Data are presented as mean \pm S.E.M. from three to seven independent experiments from densitometric scans of Western blots.

MAPk in wild-type SKNSH cells. The response was rapid (strong activation within 2 min), maximal at concentrations below 0.1 ng/ml EGF, and well-sustained 90 min after EGF. In contrast, activation of MAPk by EGF in SKNSH rMDL6 cells was both less potent (Fig. 3A) and more transient (Fig. 3B).

To determine whether the MAPk pathway in SKNSH rDOX6 cells was refractory to other stimulatory agents, the effect of PMA on MAPk activity was also assessed in wild-type and drug-resistant cells. PMA activated MAPk in SKNSH rDOX6 cells, but to a significantly lesser degree than in wild-type or SKNSH rMDL6 cells (Fig. 2C; $P < .05$, two-tailed t test).

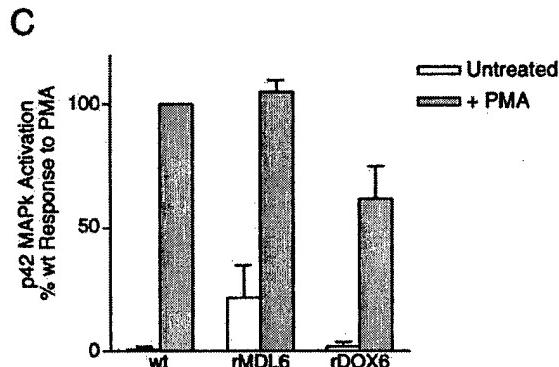
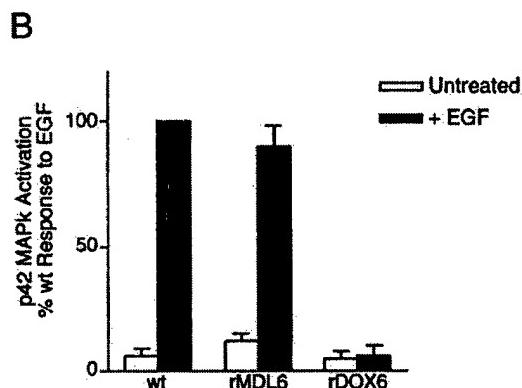
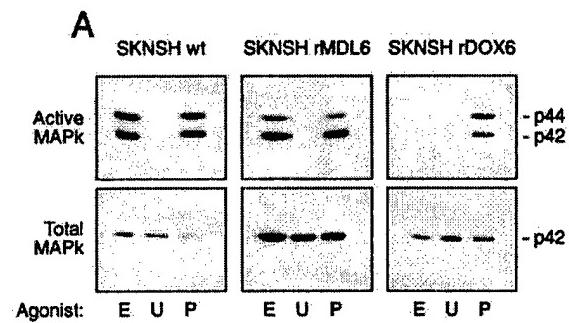


Fig. 2. Activation of MAPk by EGF and PMA in SKNSH cells. (A) SKNSH cells and drug-resistant variants were treated for 5 min with 10 ng/ml EGF (E), 200 nM PMA (P), or vehicle control (U) and processed sequentially for assay of active MAPk (upper panels) and then total MAPk (lower panels, to provide a control that confirms equivalent recovery and loading) as described in the Materials and Methods. A representative result is shown. (B and C) Quantification of the results for active, dually phosphorylated p42 MAPk by densitometric scanning. Data shown are mean \pm S.E.M. from three to five independent experiments. The response of wild-type cells to EGF or PMA was set to 100% in each experiment.

In addition to polypeptide growth factors that activate receptors with intrinsic tyrosine kinase activity, MAPk can also be strongly activated by certain agonists, such as LPA, that act through G protein-coupled pathways [26]. To

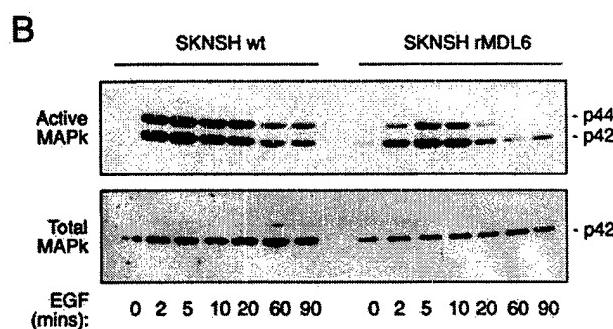
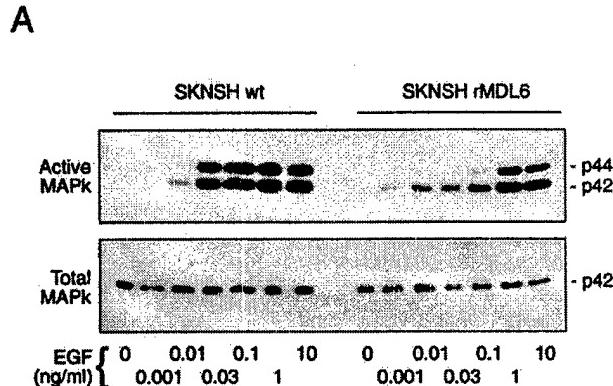


Fig. 3. Concentration-response and kinetics of the activation of MAPk by EGF in SKNSH rMDL6 cells. (A) Wild-type and SKNSH rMDL6 cells were treated for 5 min with the indicated concentrations of EGF and processed for assay of active and total ERK MAPk as described in the Materials and Methods. Data are representative of three independent experiments. (B) Wild-type and SKNSH rMDL6 cells were treated for the indicated time with 10 ng/ml EGF and processed for assay of active and total ERK MAPk as described in the Materials and Methods. Data are representative of two independent experiments.

determine whether drug resistance attenuated the response of MAPk to LPA, its effect on MAPk activation was assessed (Fig. 4). The stimulatory activity of LPA on MAPk

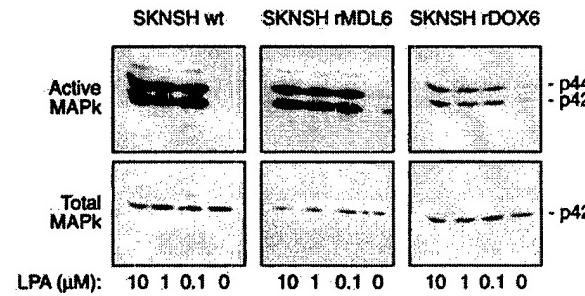


Fig. 4. LPA-dependent activation of MAPk in wild-type and drug-resistant SKNSH cells. Cells were treated for 5 min with the indicated concentrations of LPA and processed for assay of active and total ERK MAPk as described in the Materials and Methods. Data are representative of three independent experiments, which show that the response to 10 μ M LPA is maintained at 98 \pm 5% of the wild-type level in SKNSH rMDL6 cells, but significantly reduced ($P < .005$, two-tailed t test) to 58 \pm 6% of the wild-type level in the SKNSH rDOX6 line.

was similar in wild-type and SKNSH rMDL6 cells, but markedly reduced in SKNSH rDOX6 cells.

3.3. Effect of drug resistance on nuclear localization of activated MAPk

Many of the most important targets for activated MAPk are thought to be transcription factors and other nuclear proteins. Thus, the translocation of activated MAPk to the nucleus is a critical step in this signal transduction pathway [27]. To determine whether the decreases in MAPk activation observed in drug-resistant cells were accompanied by reductions in active MAPk in the nucleus, confocal immunofluorescent microscopy was performed (Fig. 5). All agonists tested, EGF, LPA, and PMA, stimulated the accumulation of active MAPk in the nuclei of wild-type SKNSH cells. However, in SKNSH rDOX6 cells, EGF was inactive and did not cause an increment in nuclear active MAPk (Fig. 5). Further, LPA was also incapable of evoking any

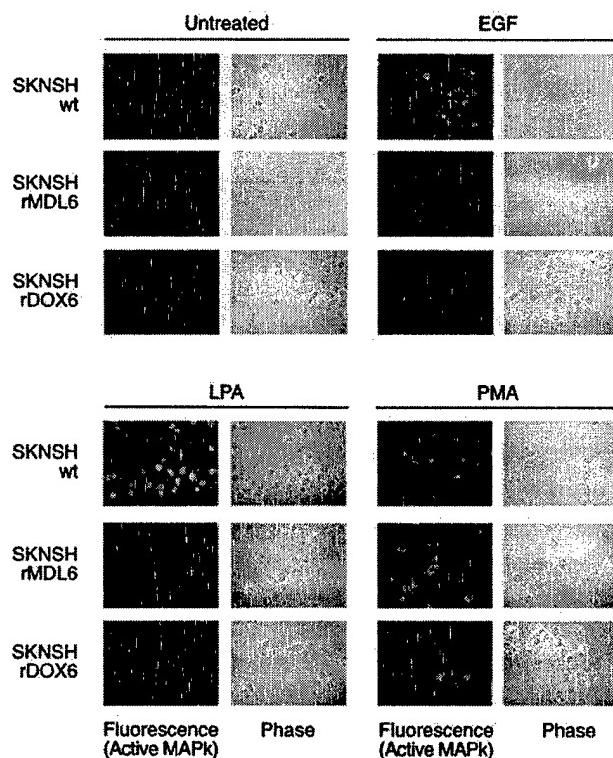


Fig. 5. Accumulation of active MAPk in the nuclei of control and stimulated SKNSH cells and drug-resistant variants. Wild-type SKNSH and the rMDL6 and rDOX6 variants were treated for 5 min with 10 ng/ml EGF, or 10 μ M LPA, or 200 nM PMA and processed for immunofluorescence of active MAPk as described in the Materials and Methods. Confocal images were obtained on a Zeiss LSM310 microscope using a $\times 63$ oil immersion lens as described [49]. Phase contrast pictures of the same field of cells at the same magnification are also shown. The apparent nuclear localization that is evident following agonist stimulation of the wild-type cells was confirmed by colocalization with the fluorescence from DAPI, a DNA marker (data not shown). Data are representative of four independent experiments.

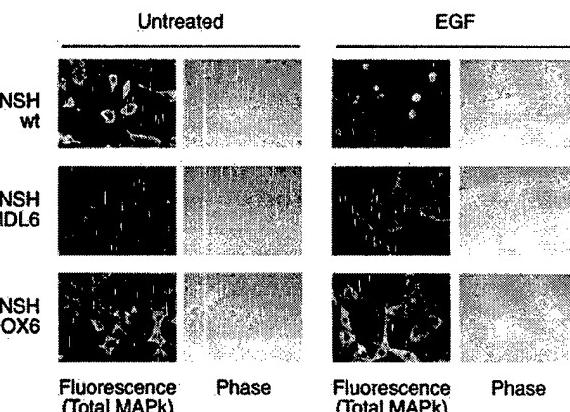


Fig. 6. EGF stimulation of nuclear translocation of MAPk in SKNSH cells. Wild-type SKNSH and the rMDL6 and rDOX6 variants were treated for 10 min with 10 ng/ml EGF and processed for confocal immunofluorescence of total MAPk as described in the Materials and Methods. Phase contrast pictures of the same field of cells at the same magnification are also shown. The apparent nuclear localization that is evident following agonist stimulation of the wild-type cells was confirmed by colocalization with the fluorescence from DAPI, a DNA marker (data not shown). Data are representative of three independent experiments.

increase in nuclear localization, despite its partial effect on MAPk activation (Fig. 4).

The results of similar experiments in SKNSH rMDL6 cells are particularly interesting. Despite the fact that EGF and LPA were able to maximally stimulate MAPk in these resistant cells (as judged by immunoblotting; Figs. 2–4), neither agonist increased the nuclear accumulation of active MAPk to the degree that occurred in wild-type cells (Fig. 5). The response of both lines of resistant cells to PMA revealed a similar pattern. Some nuclear accumulation of active MAPk was induced, but a substantial portion of the active MAPk remained in the cytoplasm.

To confirm that accumulation of active MAPk in the nuclei of wild-type SKNSH cells in response to EGF was due to translocation from the cytosol, confocal immunofluorescence for total MAPk was performed (Fig. 6). Incubation of wild-type SKNSH cells with EGF induced relocalization of nearly all MAPk from the cytosol to the nucleus. In the SKNSH rMDL6 cell line however, despite stimulation with a concentration of EGF that stimulated MAPk activity to levels obtained in wild-type cells (Fig. 3), there was little apparent relocalization of MAPk (Fig. 6). The failure of EGF to induce the relocalization of MAPk in the SKNSH rDOX6 cells would be expected from its failure to activate MAPk in this line (Fig. 2).

4. Discussion

The p44 MAPk and p42 MAPk (also termed ERK-1 and -2) are serine/threonine protein kinases that are activated by dual phosphorylation on particular threonine and tyrosine residues [28] by the enzyme termed MAPk/ERK kinase 1

(MEK1) [29] or MAPk kinase [30]. MEK1 is itself activated upon phosphorylation by upstream kinases such as Raf [31] or Mos [32]. The kinase Raf provides a link to this cascade from agonists that can induce activation of the small GTPase Ras, as Ras is known to complex with and activate Raf [33]. Both tyrosine kinase-dependent growth factor receptors [34,35] and G protein-coupled receptors [36,37] are known to activate Ras and thus the MAPk cascade. Further, activation of Raf is also a mechanism whereby phorbol esters that activate protein kinase C can lead to the stimulation of the MAPk module [38]. Once activated, MAPk can enter the nucleus and exert its effects through phosphorylation of critical substrates such as transcription factors [39]. An overview of this pathway illustrating perturbations in the kinase cascade that occur in drug-resistant SKNSH cells is presented in Fig. 7.

The results demonstrate that drug-resistant SKNSH cells manifest decreases in both the activation of MAPk and its subsequent nuclear translocation. This reduction in MAPk signaling of drug-resistant cells in response to EGF can be only partially explained by a reduction in the number of functional EGFRs. Modulation of EGFR expression in drug-resistant neuroblastoma cells [24] and during the progression of various human tumors has been noted and suggested to contribute to the transformed phenotype [40]. However, it is also clear from the present studies on SKNSH rMDL6 cells that, even under conditions where maximal phosphorylation and activation of MAPk has been achieved

in response to EGF, this was not sufficient to induce nuclear relocalization of MAPk to the extent that occurs in wild-type cells (Figs. 5 and 6). The kinetics of MAPk activation, with sustained phosphorylation favoring relocalization to the nucleus, have previously been identified as critical parameters that determine translocation from the cytosol [41]. Thus, it may be that the more transient activation of MAPk occurring in EGF-stimulated SKNSH rMDL6 cells (Fig. 3B) prevents significant accumulation of active nuclear MAPk.

The reduced effectiveness of LPA and PMA in stimulating the translocation of active MAPk to the nuclei of drug-resistant cells would further suggest that there is a defect in the MAPk signaling pathway between generation of the active MAPk and its translocation to the nucleus. Such a defect has recently been described to occur as cells enter senescence [42]. Since we have evidence that the induced nuclear translocation of other signaling components, such as phospho-Stat3, is not compromised in the drug-resistant cells [43], it is unlikely that there is a broad deficit in nuclear trafficking. One possibility is that there may be induction of a selective phosphatase with affinity for MAPk in drug-resistant lines that curtails activation and prevents translocation of the enzyme to the nucleus. Such a situation occurs following expression of the ret oncogene in PC12 cells [44].

What is the relevance of decreased growth factor-stimulated MAPk activity to cytotoxic drug resistance in neuroblastoma cells? In most transformed systems, increased activation of the MAPk module [45] has been associated with a more malignant phenotype [46]. This is in accord with the known ability of the Ras/MAPk system to induce cell cycle progression and division [47]. The reduced proliferation rate of the drug-resistant cell lines may therefore be secondary to decreased MAPk signaling. Thus, it may be that a slower rate of cell cycle progression affords drug-resistant cells increased opportunity to repair drug-induced damage. Should such a mechanism be substantiated in subsequent work, then it may have implications for future design of therapeutic regimens. If reduced MAPk signaling is found to be more generally associated with acquired drug resistance, then agents that inhibit the MAPk cascade may potentially exert unintended protection from other codelivered cytotoxic agents.

A further important consequence of reduced MAPk signaling may be of particular relevance for neuroblastoma and other neuroectodermal tumors and cell lines, which reflect a failure of cellular differentiation mechanisms [48]. In the PC12 phaeochromocytoma line, for example, sustained activation of the MAPk cascade by NGF produces differentiation rather than proliferation [41]. In contrast, proliferative growth signals in PC12 cells, such as those stimulated by EGF, may be encoded through transient MAPk activation [41]. Further, prevention of the NGF-dependent accumulation of active MAPk in the nucleus of PC12 cells by the ret oncogene blocks differ-

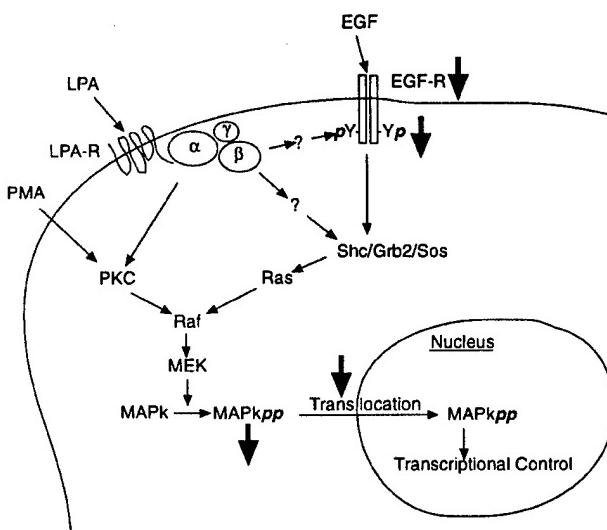


Fig. 7. Drug resistance and MAPk signal transduction in SKNSH neuroblastoma cells. Several defects in the MAPk signaling pathway have been found in the drug-resistant SKNSH lines (indicated by the large arrows). First, there is a decrease in the expression and phosphorylation of EGFR. There are no apparent decreases in certain other intermediaries in this pathway such as Shc or Sos (data not shown) or MAPk itself (Fig. 2). Second, the active, dually phosphorylated form of MAPk is diminished. Third, the translocation of the active MAPk to the nucleus is reduced. Further discussion of the implications of these results is in the text.

entiation [44]. Thus, it seems plausible to suggest that decreased translocation of active MAPK to the nuclei of drug-resistant SKNSH cells may also confer continued progression of these transformed cells to an increasingly de-differentiated phenotype.

Acknowledgments

We thank Sandra H. Clark and Irina Laer for technical assistance, Eric N. March for data quantification, and Roberta Gerard for preparation of the manuscript. This work was supported by the Anderson Foundation and the Medical Research Institute Council (to B.L.M.) and a Research Starter Grant and a Faculty Development Award from the PhRMA Foundation and Grant No. DAMD17-00-1-0544 from the Department of the Army (to R.R.M.). Confocal facilities were supported in part by the EHS Center in Molecular and Cellular Toxicology with Human Applications (P30 ES-06639). We thank Linda Mayernik and Kamiar Moin for their assistance with the immunofluorescence. The content of the information does not necessarily reflect the position or policy of the US Government, and no official endorsement should be inferred.

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